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Distribution of Conjugative-Plasmid-Mediated 16S rRNA Methylase Genes among Amikacin-Resistant *Enterobacteriaceae* Isolates Collected in 1995 to 1998 and 2001 to 2006 at a University Hospital in South Korea and Identification of Conjugative Plasmids Mediating Dissemination of 16S rRNA Methylase[▽]

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The distribution of conjugative-plasmid-mediated 16S rRNA methylase genes among amikacin-resistant *Enterobacteriaceae* collected between 1995 and 1998 and between 2001 and 2006 at a university hospital in South Korea was examined, and conjugative plasmids carrying the 16S rRNA methylase genes were characterized by PCR-based replicon typing and by determination of their antimicrobial resistance pattern. Among the 7,127 isolates, 463 isolates showed a high level of resistance to amikacin, and 218 of the 463 isolates transferred amikacin resistance by conjugation. Among the 218 isolates, *armA* was detected in 153 isolates (88 *Klebsiella pneumoniae*, 28 *Escherichia coli*, 19 *Enterobacter cloacae*, and 6 *Serratia marcescens* isolates and 12 isolates of other organisms), and *rmtB* was detected in 51 isolates (32 *K. pneumoniae* isolates, 18 *E. coli* isolates, and 1 *Citrobacter freundii* isolate). The first appearance of *armA* was in 1997. The *armA* gene was carried by conjugative plasmids of replicon groups IncL/M, IncFIIAs, IncF, IncA/C, IncHI2, and Inc(unidentified) in 38, 20, 7, 9, 4, and 75 strains, respectively. The *rmtB* gene was carried by conjugative plasmids of groups IncA/C, IncF, and IncI1-Iγ in 43 strains, 7 strains, and 1 strain, respectively. Transconjugants that received the IncL/M plasmid carrying *armA* or the IncA/C plasmid carrying *rmtB* showed an additional resistance to cefotaxime. Transconjugants that received the IncFIIA plasmid or Inc(unidentified) plasmid carrying the *armA* gene showed an additional resistance to cefoxitin and a high MIC_{50} (0.25 mg/liter) of ciprofloxacin. In conclusion, this study demonstrated that the dissemination of 16S rRNA methylase genes among the *Enterobacteriaceae* is mediated by conjugative plasmids of various incompatibility groups that confer resistance to multiple drugs, including aminoglycosides, extended-spectrum β-lactams, and/or quinolones.

Aminoglycosides have a high affinity for the 16S rRNA of the bacterial 30S ribosome, and they block protein synthesis (14, 20). Over the past few decades, there have been many studies conducted regarding the mechanisms of resistance to aminoglycosides. One of the most common mechanisms of resistance to aminoglycosides is the production of aminoglycoside-modifying enzymes, such as 1-*N*-aminoglycoside acetyltransferase (AAC), adenyltransferase, and phosphotransferase (14, 20). Amikacin was developed to suppress a variety of aminoglycoside-modifying enzymes from their accessing target sites (12), and therefore rare, amikacin-resistant bacteria could be expected. Recently, a series of special methylases that protect microbial 16S rRNA, however, were identified in several nosocomial pathogens, and these enzymes are capable of conferring extraordinarily high levels of resistance (MIC > 512 mg/liter) to most clinically important aminoglycosides, including amikacin, isepamicin, arbekacin, kanamycin, tobramycin, and gentamicin (5–8, 21–24). Since the first identification of a gene encoding 16S rRNA methylase, *rmtA*, from a *Pseudomonas aeruginosa* isolate in 2003 (20), four major 16S rRNA methylases, *rmtA* (24), *armA* (6), *rmtB* (5), and *rmtC* (21), have been reported to occur in several nosocomial pathogens, including *P. aeruginosa*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Proteus mirabilis* (5, 6, 13, 21, 24). The genes for 16S rRNA methylases are mediated by mobile genetic elements that are carried by transferable large plasmids (5, 7–8); thus, the spread of these genes among gram-negative bacilli has been concerning. Recently, several studies have demonstrated the dissemination of the *armA* gene to various species of *Enterobacteriaceae* in European countries (7), the spread of the multidrug-resistant *Escherichia coli* and *K. pneumoniae* isolates that produce both extended-spectrum β-lactamases (ESBLs) and 16S rRNA methylases in Taiwan (23), the dissemination of 16S rRNA methylase-mediated amikacin-resistant isolates of *K. pneumoniae* and *A. baumannii* in South Korea (13), and the emergence of 16S rRNA methylases in Belgium (2).

In this study, we tried to determine the beginning of the emergence and the current prevalence of plasmid-mediated 16S rRNA methylases among the *Enterobacteriaceae.* This was done by detecting the 16S rRNA methylase genes in amikacin-resistant *Enterobacteriaceae* isolates that were collected in 1995

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TABLE 1. Numbers of isolates studied and rates of resistance to amikacin among *Enterobacteriaceae* that were collected from a university hospital in South Korea between 1995 and 2006

Species	No. of isolates studied (no. of isolates resistant to amikacin) in:									Total no. of isolates (total no. resistant to amikacin)	Rate of resistance to amikacin (%)
	1995	1996	1997	1998	2001 to ~2002	2003	2004	2005	2006		
E. coli	132 (0)	197 (5)	157 (1)	6 (2)	492 (13)	156 (9)	418 (14)	908 (31)	860 (25)	3,326 (100)	3.0
K. pneumoniae	60 (2)	61 (3)	141 (3)	42 (3)	304 (8)	83 (5)	226 (26)	605 (111)	537 (108)	2,059 (269)	13.1
Klebsiella spp.	2 (0)	9 (0)	24 (2)	4 (0)	39 (0)	12 (0)	18 (0)	36 (2)	5 (1)	149 (5)	3.4
E. cloacae	27 (4)	25 (3)	29 (9)	5 (2)	86 (5)	13 (2)	65 (6)	109 (6)	125 (5)	484 (42)	8.7
Enterobacter spp.	5 (0)	7 (1)	29 (0)	4 (2)	45 (0)	5 (0)	44 (0)	107 (2)	108 (5)	354 (10)	2.8
S. marcescens	16 (0)	9 (0)	43 (2)	7 (0)	75 (5)	45 (7)	23 (2)	38 (1)	51 (6)	307 (23)	7.5
Serratia spp.								2 (0)	2 (0)	4 (0)	0
C. freundii	4 (0)	2 (0)	8 (1)	1 (1)	15 (0)	14 (2)	17 (0)	47 (3)	53 (1)	161 (8)	5.0
Citrobacter spp.							3 (0)	6 (0)	4 (1)	13 (1)	2.6
Morganella morganii	4 (0)	2 (0)	4 (0)		10 (0)	4 (0)	25 (1)	2 (1)	25 (0)	76 (2)	3.2
P. mirabilis	6 (1)	3 (0)	6 (0)	3 (0)	18 (0)	10 (0)	15 (0)	39 (1)	40 (1)	140 (3)	2.1
Proteus spp.	2 (0)	1 (0)	2 (0)		5 (0)	2 (0)	4 (0)	11 (0)	16 (0)	43 (0)	0
Providencia spp.	3 (0)				3 (0)		2 (0)	3 (0)		11 (0)	0
Total no. of isolates (total no. resistant to amikacin)	261 (7)	316 (12)	443 (18)	72 (10)	1,092 (31)	344 (25)	860 (49)	1,913 (158)	1,826 (153)	7,127 (463)	
Rate of resistance to amikacin (%)	2.7	3.8	4.1	13.9	2.8	7.3	5.7	8.3	8.4	6.5	

to 1998 and 2001 to 2006 at a university hospital in South Korea. In addition, in order to understand how the genes were disseminated, conjugative plasmids carrying the 16S rRNA methylase genes were identified by PCR-based replicon typing of the major plasmid incompatibility (Inc) groups among the *Enterobacteriaceae*.

MATERIALS AND METHODS

Bacterial strains. A total of 7,217 nonduplicate *Enterobacteriaceae* isolates were collected in 1995 to 1998 and 2001 to 2006 from patients hospitalized at Kyungpook National University (KNU) Hospital in South Korea. Bacterial species were identified by using the Vitek GNI system (bioMerieux, Marcy L'Etoile, France) or the API 20E kit (bioMerieux, Marcy L'Etoile, France). All isolates were tested for resistance to amikacin using the agar dilution method (4).

Transfer of the amikacin resistance determinant by conjugation. In order to test the transferability of the amikacin resistance determinant to the rifampin-resistant *E. coli* strain RG 488 and the azide-resistant strain J53, all amikacin-resistant isolates were included as putative donors in a conjugation assay using the broth mating method. The transconjugants were selected on a Muller-Hinton agar plate supplemented with amikacin (64 μg/ml) and rifampin (50 μg/ml) or sodium azide (200 μg/ml).

Detection of 16S rRNA methylase genes and *aacA4*. A PCR method was used to detect the genes encoding 16S rRNA methylases (*rmtA*, *rmtB*, and *armA*) and the gene encoding AAC(6′)-Ib (*aacA4*), which are known to confer resistance to amikacin. The primers used were those described by Yan et al. (23) for *rmtA*, *rmtB*, and *armA* and by Shi et al. (18) for *aacA4*. PCRs were performed as described previously (18, 23).

PCR-based replicon typing. All transconjugants obtained from a conjugation assay were subjected to typing by a PCR method, based on replicons of the major plasmid incompatibility groups among *Enterobacteriaceae*, that was developed by Carattoli et al. (3). The plasmid DNAs from the transconjugants were isolated by an alkaline lysis method (1) and amplified by five multiplex and three simplex PCRs using 18 pairs of primers that recognized Inc replicons FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA. As positive controls in the PCRs, R27 (HI1), R478 (HI2), R483 (I1), R446b (L/M), RN3 (N), Tp181 (FIme), RS-a (W), RP4(P), R40a (A/C), Rts1 (T), R124 (FIV), R387 (K), R16 (B/O), and R6K (X) were used.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing and the determination of MICs were performed by the agar dilution method (4). The antimicrobial agents included the following: gentamicin and kanamycin (Duchefa, Haarlem, The Netherlands), amikacin and trimethoprim (ICN Biomedicals, Irvine, CA), streptomycin (Sigma Chemical Co., St. Louis, MO), ampicillin (USB, Cleveland, OH), cefoxitin (Sigma), cefotaxime (Sigma), cefepime (Boryung Inc., Seoul, South Korea), aztreonam (Sigma), ceftazidime (Sigma), ciprofloxacin (Fluka, Buchs, Switzerland), chloramphenicol (Sigma), tetracycline (Sigma), and sulfamethoxazole (Sigma). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains.

RESULTS

Among the 7,217 clinical isolates of *Enterobacteriaceae* that were collected from the KNU Hospital in South Korea in 1995 to 1998 and 2001 to 2006, 463 (6.5%) strains showed resistance to amikacin (Table 1). The rate of resistance to amikacin increased from 2.7% in 1995 to 8.4% in 2006. Overall, 269 (13.1%) of the 2,059 *Klebsiella pneumoniae*, 100 (3.0%) of the 3,326 *Escherichia coli*, 42 (8.7%) of the 484 *Enterobacter cloacae*, and 23 (7.5%) of the 307 *Serratia marcescens* isolates were resistant to amikacin. The rates of resistance to amikacin in *Citrobacter freundii*, *Morganella morganii*, and *Proteus mirabilis* were 5.0% (8/161 isolates), 3.2% (2/76 isolates), and 2.1% (3/140 isolates), respectively.

By a conjugation experiment, amikacin resistance could be transferred to 218 (47.1%) of the 463 amikacin-resistant isolates. All 218 strains and their transconjugants were examined for the presence of the genes that encode 16S rRNA methylases (*armA*, *rmtA*, and *rmtB*) and the gene that encodes AAC(6′)-Ib (*aacA4*). Among the 218 strains, *armA*, *rmtB*, and

TABLE 2. Distribution of *aacA4* and 16S rRNA methylase genes among the 218 amikacin-resistant isolates of *Enterobacteriaceae* that were collected from a university hospital in South Korea

Yr of isolation	No. of strains carrying the following gene(s):						Total
	aacA4 alone	*armA* alone	*armA* and *aacA4*	*rmtB* alone	*rmtB* and *aacA4*	None[a]	
1995	2						2
1996	9						9
1997	1	7	2				10
1998	1	3	1				5
2001		4		2	5		11
2002		1		5			6
2003			9				9
2004		19	6	1			26
2005		45	8	20	1	1	75
2006		24	24	17			65
Total no. of strains	13	103	50	45	6	1	218

[a] No gene was detected.

TABLE 3. Distribution of *aacA4* and 16S rRNA methylase genes among the 218 amikacin-resistant isolates of *Enterobacteriaceae* collected from a university hospital in South Korea between 1995 and 2006

Species	No. of strains carrying the following amikacin resistance gene(s):						Total no. of strains
	aacA4 alone	*armA* alone	*armA* and *aacA4*	*rmtB* alone	*rmtB* and *aacA4*	None[a]	
E. coli	5	14	14	17	1	1	52
K. pneumoniae	3	65	23	27	5		123
Klebsiella spp.		1	1				2
E. cloacae	4	17	2				23
Enterobacter spp.	1	5	1				7
S. marcescens			6				6
C. freundii			3	1			4
M. morganii		1					1
Total no. of strains	13	103	50[b]	45	6[c]	1	218

[a] No gene was detected.
[b] Thirteen of 50 strains transferred the *armA* gene alone to the recipient *E. coli* strain, as determined by the conjugation assay.
[c] All six strains transferred the *rmtB* gene alone to the recipient *E. coli* strain, as determined by the conjugation assay.

aacA4 were detected in 153 (33.1%), 51 (11.0%), and 69 (31.7%) isolates, respectively, but *rmtA* was not detected in any of the isolates (Tables 2 and 3). *aacA4* was detected with *armA* (50 strains) or *rmtB* (6 strains), and it was transferred simultaneously with *armA* (37 strains) to the recipient *E. coli* strain, but not with *rmtB*. The first appearances of *armA* and *rmtB* were in 1997 and 2001, respectively (Table 2). Whereas the *armA* gene was detected among various species of *Enterobacteriaceae*, *rmtB* was detected only in *E. coli*, in *K. pneumoniae*, and in one *Citrobacter freundii* isolate (Table 3).

In order to identify the conjugative plasmids carrying *armA* or *rmtB*, PCR-based replicon typing was performed using 18 pairs of primers that recognized the Inc replicons FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA (Table 4). IncL/M, IncFIIAs, and IncHI2 were detected in 37 (24.2%), 20 (13.1%), and 4 (2.6%) of the 153 transconjugants that carry *armA*, respectively, but not in the transconjugants that carry *rmtB*. IncA/C was detected in 41 (80.3%) of the 51 transconjugants that carry *rmtB* and in 7 (4.6%) of the 153 transconjugants that carry *armA*. IncF was detected in 6 (11.7%) of the 51 transconjugants that carry *rmtB* and in 7 (4.6%) of the 153 transconjugants that carry *armA*. IncFIB and IncB/O were detected only in the transconjugants that carry *aacA4* alone. In 75 (49.0%) of the 153 transconjugants that carry *armA*, none of the 18 replicons were detected. Replicons such as N, P, W, T, K, and X were not detected in any of the strains that carry *armA*, *rmtB*, or *aacA4*.

MICs of various kinds of antimicrobial agents for the transconjugants that received plasmids carrying *armA* or *rmtB* were determined. All transconjugants showed high levels of resistance (MICs, >256 μg/ml) to amikacin, kanamycin, and gentamicin. MIC_{50}s of 12 kinds of antimicrobial agents other than aminoglycosides were calculated among the strains that were grouped with regard to the replicon types of plasmid incompatibility groups and the presence of *armA* or *rmtB* (Table 5). Groups with fewer than five strains were excluded from the calculation of MIC_{50}s. Surprisingly, a very distinctive antimicrobial resistance pattern was revealed in each group. Compared to other groups, transconjugants that received the IncL/M plasmid carrying *armA* and the IncA/C plasmid carrying *rmtB* showed higher MIC_{50} values for cefotaxime (64 μg/ml) and cefepime (8 μg/ml). In the transconjugants that received the Inc(unidentified) plasmid carrying *armA*, higher MIC_{50} values of aztreonam (32 μg/ml), ceftazidime (32 μg/ml), cefoxitin (32 μg/ml), and ciprofloxacin (0.25 μg/ml) were revealed. Transconjugants that received the IncFIIAs plasmid carrying *armA* showed higher MIC_{50} values of cefoxitin (32 μg/ml) and ciprofloxacin (0.25 μg/ml). Transconjugants that received the IncA/C plasmid carrying *rmtB* showed additional resistance to multiple antimicrobial agents, including ampicillin, cefotaxime, chloramphenicol, streptomycin, sulfamethoxazole, trimethoprim, and tetracycline.

The year of isolation and the species of the *Enterobacteriaceae* isolates that were able to transfer *armA* or *rmtB* to the recipient *E. coli* isolate are shown in Table 6. Whereas 9 of the 13 strains that were isolated from 1997 and 1998 were *Enterobacteriaceae*, including 5 *Enterobacter cloacae* strains, 2 *Citrobacter freundii* strains, 1 *Klebsiella oxytoca* strain, and 1 *Enterobacter agglomerans* strain, 162 of the 191 strains that were isolated after 2001 were *E. coli* and *K. pneumoniae*. The major conjugative plasmids carrying *armA* were IncA/C and IncHI2 plasmids until 1998, but after 2001, they were replaced by the conjugative plasmids of incompatibility groups IncFIIAs, IncL/M, and Inc(unidentified). Between 2003 and 2004, a rapid increase in the number of IncL/M and Inc(unidentified) conjugative plasmids carrying *armA* among the *Enterobacteriaceae*, especially in *K. pneumoniae*, was revealed. Among the *K. pneumoniae* isolates, the *armA*-carrying conjugative plasmids of groups Inc(unidentified), IncL/M, IncFIIAs, IncF, and IncA/C were detected in 55, 17, 11, 3, and 2 isolates, respectively. Among the *E. coli* isolates, the *armA*-carrying conjugative plasmids of groups IncL/M, Inc(unidentified), IncFIIAs, IncF, and IncA/C were detected in 10 isolates, 9 isolates, 3 isolates, 2 isolates, and 1 isolate, respectively. Among the *E. cloacae* iso-

TABLE 4. Incompatibility groups of conjugative plasmids which confer a high level of resistance to amikacin due to the presence of the 16S rRNA methylase gene and/or *aacA4* gene

Incompatibility group(s)	No. of transconjugants carrying the following gene(s):					Total	No. (%) of transconjugants with:		*P* value[c]
	aacA4 alone	*armA* alone	*armA* and *aacA4*	*rmtB* alone	None[a]		*armA*	*rmtB*	
FIIAs							20 (13.1)		0.007
HI2		4				4	4 (2.6)		
L/M		8	29			37	37 (24.2)		<0.001
L/M, F			1			1	1 (0.7)		
A/C	2	4	3	41	1	51	7 (4.6)	41 (80.3)	<0.001
A/C, I1-Iγ		1	1			2	2 (1.3)		
A/C, F				1		1		1 (2.0)	
A/C, F, FIA				1		1		1 (2.0)	
F		7		6	1	14	7 (4.6)	6 (11.7)	0.069
F, Y				1		1		1 (2.0)	
I1-Iγ				1		1		1 (2.0)	
F, FIB	1					1			
B/O	4					4			
NI[b]	4	72	3		1	80	75 (49.0)		<0.001
Total no. of transconjugants	11	116	37	51	3	218	153	51	

[a] No gene was detected.
[b] NI, not identified.
[c] The chi-square test was performed.

lates, the *armA*-carrying conjugative plasmids of groups IncFIIAs, Inc(unidentified), IncHI2, IncL/M, and IncF were detected in six, six, three, two, and two isolates, respectively. The IncL/M conjugative plasmid carrying *armA* was detected in six of six *S. marcescens* isolates, and the IncA/C conjugative plasmid carrying *armA* was detected in three of the four *C. freundii* isolates. The IncA/C conjugative plasmid carrying *rmtB* was detected in 31 *K. pneumoniae* isolates, 11 *E. coli* isolates, and 1 *C. freundii* isolate. The IncF conjugative plasmid carrying *rmtB* was detected in six *E. coli* isolates and one *K. pneumoniae* isolate. The IncI1-Iγ conjugative plasmid carrying *rmtB* was detected in one *E. coli* isolate.

DISCUSSION

We have studied the distribution of conjugative-plasmid-mediated 16S rRNA methylase genes, such as *armA*, *rmtA*, and *rmtB*, among the amikacin-resistant *Enterobacteriaceae* that were collected in 1995 to 1998 and 2001 to 2006 at KNU Hospital in South Korea. In addition, the conjugative plasmids that are responsible for disseminating the 16S rRNA methylase genes were identified by replicon typing of plasmid incompatibility groups. The results indicate that the widespread presence of *armA* and *rmtB* among amikacin-resistant *Enterobacteriaceae* has been mediated by conjugative plasmids of various

TABLE 5. Antimicrobial susceptibilities of transconjugants grouped by presence of the *armA* or *rmtB* gene and by plasmid incompatibility group[a]

Antimicrobial agent	MIC_{50} for isolates with the indicated gene and Inc plasmid[b]:						
	armA (*n* = 143)					*rmtB* (*n* = 47)	
	FIIAs (*n* = 20)	L/M (*n* = 37)	A/C (*n* = 7)	F (*n* = 7)	NI (*n* = 75)	A/C (*n* = 41)	F (*n* = 6)
Ampicillin	512	>512	>512	>512	>512	>512	>512
Aztreonam	<1	8	<1	<1	32	8	<1
Ceftazidime	<1	2	<1	<1	32	2	<1
Cefotaxime	<1	64	<1	<1	4	64	<1
Cefepime	<1	8	<1	<1	<1	8	<1
Cefoxitin	32	4	1	2	32	4	4
Ciprofloxacin	0.25	0.03	0.015	0.03	0.25	0.015	0.25
Chloramphenicol	4	4	64	4	4	128	4
Streptomycin	256	4	512	256	256	256	4
Sulfamethoxazole	16	16	>512	16	16	>512	16
Trimethoprim	<1	<1	<1	8	<1	>512	<1
Tetracycline	<1	<1	1	<1	<1	32	<1

[a] Groups with fewer than five strains were excluded from calculations of MIC_{50}s.
[b] Numbers of strains included in each group are shown in parentheses. NI, not identified.

TABLE 6. Years of isolation and species of *Enterobacteriaceae* isolates which were able to transfer the plasmids of various incompatibility groups carrying *armA* or *rmtB*

Yr of isolation and species[a]	No. of strains that transfer a conjugative plasmid of the indicated Inc group carrying gene:								
	armA						*rmtB*		
	FIIAs	HI2	L/M	A/C	F	NI[b]	A/C	F	I1-Iγ
1997–1998									
E. coli				3[d]	1				
K. pneumoniae									
Others		4		3		2			
2001									
E. coli								1	
K. pneumoniae						1	6		
Others	3								
2002									
E. coli							4[e]	1[g]	
K. pneumoniae									
Others	1								
2003									
E. coli			2						
K. pneumoniae			1						
Others			6						
2004									
E. coli	1		1	1		2	1		
K. pneumoniae	1		1		2	10			
Others					1	5			
2005									
E. coli	1		1		1	5	4[f]	4	1
K. pneumoniae	6		2	1	1	30	10	1	
Others	2		1		1	1	1		
2006									
E. coli	1		6			2	2		
K. pneumoniae	4		13[c]	1		14	15		
Others			4			3			
Total no. of strains	20	4	38[c]	9[d]	7	75	43[e,f]	7[g]	1

[a] Others, *Enterobactericeae* isolates other than *E. coli* and *K. pneumoniae*, including 19 *E. cloacae* isolates, 6 *Enterobacter* species isolates, 6 *S. marcescens* isolates, 4 *C. freundii* isolates, 2 *K. oxytoca* isolates, and 1 *M. morganii* isolate.
[b] NI, not identified.
[c] One isolate was positive for L/M and F.
[d] Two isolates were positive for A/C and I1-Iγ.
[e] One isolate was positive for A/C and F.
[f] One isolate was positive for A/C, F, and FIA.
[g] One isolate was positive for F and Y.

incompatibility groups conferring additional resistance to other kinds of antimicrobial agents, including ESBLs and quinolones.

The *armA* gene was initially sequenced from a *C. freundii* strain that was isolated in Poland (GenBank accession no. AF550415) and later characterized from a *K. pneumoniae* strain that was isolated in 2000 from a patient in France (6). The *rmtB* gene was first identified in *S. marcescens* S-95 isolated in 2002 from a patient in Japan (5). In the current study, the *armA* gene was first detected among three *E. coli* strains, three *E. cloacae* strains, one *C. freundii* strain, and one *K. oxytoca* strain that were isolated in 1997. The year of the first appearance of *rmtB* could not be correctly determined due to the exclusion of isolates distributed in 1999 and 2000; however, the emergence of *rmtB* as early as 2001 was assumed because *rmtB* was detected in seven strains that were isolated in 2001. Thus, this study has demonstrated that *armA* and *rmtB* emerged earlier than what has been reported so far.

The overall prevalence rates of *armA* and *rmtB* among the *Enterobacteriaceae* at KNU Hospital were 2.1% (153/7,127 isolates) and 0.7% (51/7,127 isolates), respectively. The overall prevalence rates of *armA* were 4.3% (88/2,059 isolates) for *K. pneumoniae*, 3.9% (19/484) for *E. cloacae*, 2.0% (6/307) for *S. marcescens*, 2.0% (3/161) for *C. freundii*, and 0.8% (28/3,326) for *E. coli*, thus indicating that *armA* is widespread among the various species of *Enterobacteriaceae*. The overall prevalence rates of *rmtB* were 1.6% (32/2,059 isolates) for *K. pneumoniae* and 0.5% (18/3,326) for *E. coli*, indicating the limited spread of *rmtB* among the *Enterobacteriaceae* compared to the spread of *armA*. The prevalence rates of *armA* and *rmtB* among *K. pneumoniae* and *E. coli* were much higher than those reported in a previous study performed in Taiwan (23), in which the prevalence rates were 0.9% (15/1,624 isolates) and 0.3% (5/1,624) for *K. pneumoniae* and 0.4% (10/2,559) and 0.04% (1/2,559) for *E. coli*, respectively.

The dissemination mechanisms of the genes that encode 16S rRNA methylases are of clinical significance since the genes confer a high level of resistance to all clinically available aminoglycosides, except streptomycin, and they were often linked to other resistance determinants, such as $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-3}}$, $bla_{\text{CTX-M-14}}$, *sul1*, and *dfrXII* (2, 7). From studies of the genetic environments of *armA* and *rmtB*, the *armA* gene was part of functional composite transposon Tn*1548* in plasmid pIP1204 (7) and the *rmtB* gene was found in the flanking region of the Tn*3*-like structure (5), suggesting that the spread of *armA* and *rmtB* was by transposition. Another mechanism, the dissemination of *armA* and *rmtB* by conjugative plasmids, has been demonstrated in a few studies: *armA* by a broad-host-range IncL/M conjugative plasmid (2, 5, 7, 23) and by a self-transferable IncN plasmid in an *E. coli* pig isolate from Spain (9) and *rmtB* by an IncF1 plasmid (2). The present study, however, demonstrated that conjugative plasmids that belonged to a variety of incompatibility groups were involved in the dissemination of *armA* and *rmtB* among *Enterobacteriaceae*. *armA* was carried by IncA/C and IncHI2 conjugative plasmids until 1998 but, after 2001, carried by plasmids of groups Inc(unidentified), IncL/M, IncFIIAs, and IncF. *rmtB* was carried by IncA/C, IncF, and IncI1-Iγ plasmids. In addition, the major plasmids that were responsible for the dissemination of *armA* and *rmtB* were completely different in terms of the incompatibility groups of the plasmids. The incompatibility groups of major conjugative plasmids that were involved in the dissemination of *armA* were Inc(unidentified) (75/153 isolates [49.0%]), IncL/M (37/153 isolates [24.2%]), and IncFIIAs (20/153 isolates [13.1%]), whereas the major conjugative plasmids that were involved in the dissemination of *rmtB* were IncA/C (41/51 isolates [80.3%]) and IncF (6/51 isolates [11.7%]). This finding suggests that the dissemination of *armA* and *rmtB* among *Enterobacteriaceae* isolates has been independently carried out by conjugative plasmids of different incompatibility groups. This suggestion could be supported with another finding, namely, that *armA* was detected among various species of *Enterobacteriaceae* but that *rmtB* was detected only in *E. coli* and *K. pneumoniae*, as well as in one *C. freundii* isolate. Interestingly, the *aacA4* gene, which was the main amikacin resistance determinant before the emergence of *armA* and *rmtB*, was identified along with *armA* or *rmtB* and transferred simultaneously with *armA* but not *rmtB*. An IncL/M plasmid was detected in most of the transconjugants that carried both *aacA4* and *armA*, suggesting the colocalization of *aacA4* and *armA* on a broad-host-range IncL/M conjugative plasmid. Localization of *aacA4* on an IncL/M plasmid that was carried by gentamicin- and

amikacin-resistant *Salmonella enterica* serotype Typhimurium isolates has been reported in a previous study (19), and the localization of *armA* on an IncL/M plasmid has been reported repeatedly (2, 5, 7, 23). However, the colocalization of *aacA4* and *armA* on an IncL/M plasmid has never before been demonstrated.

It is very interesting to note that a distinctive antimicrobial resistance pattern is revealed in each group whose transconjugants were grouped by the presence of *armA* or *rmtB* and by a plasmid incompatibility group, suggesting the linkage of *armA* and *rmtB* to other plasmid-located antimicrobial resistance genes and the association of these genes with specific plasmid backbones. The *armA*-carrying IncL/M plasmid and the *rmtB*-carrying IncA/C plasmid were associated with resistance to cefotaxime but not to ceftazidime, strongly suggesting the presence of $bla_{\text{CTX-M}}$. These results were in agreement with those of a previous study in which the association of *armA* with $bla_{\text{CTX-M-3}}$ on the IncL/M plasmid was demonstrated (7). The association of *rmtB* with $bla_{\text{CTX-M-14}}$ was also demonstrated in a previous study (2), but the conjugative plasmid that carried these genes was the IncF plasmid. Besides being associated with resistance to cefotaxime, the *rmtB*-carrying IncA/C plasmids were associated with additional resistance to multiple drugs, including chloramphenicol, streptomycin, sulfamethoxazole, trimethoprim, and tetracycline, suggesting the presence of an integron. The *armA*-carrying Inc(unidentified) and the IncFIIAs plasmids were associated with resistance to cefoxitin, suggesting the presence of plasmid-mediated AmpC-type β-lactamase (pACBL). A similar association between *armA* and pACBL, such as with CMY-2 or DHA-1, was reported in a previous study (13). The *armA*-carrying IncFIIAs and Inc(unidentified) plasmids and *rmtB*-carrying IncF plasmid were associated with a relatively high MIC_{50} of ciprofloxacin (0.25 mg/liter), suggesting the presence of the plasmid-mediated quinolone resistance determinant *qnr*. Although the association between *qnr* determinants and ESBL or pACBL has been demonstrated in previous reports (10, 15–17), the connection of *qnr* determinants with *armA* or *rmtB* has not yet been determined. In fact, transconjugants that received an Inc(unidentified) plasmid carrying *armA* showed additional resistance to oxyimino-cephalosporins, such as aztreonam and ceftazidime, suggesting the production of ESBL. Therefore, the association of *armA*, pACBL, *qnr*, and ESBL on the conjugative Inc(unidentified) plasmid is suggested, and a high rate of such a conjugative plasmid among *Enterobacteriaceae* isolated after 2004 threatened the spread of multidrug-resistant *Enterobacteriaceae*, which are resistant to almost all clinically important antimicrobial agents, such as aminoglycosides, ESBLs, and quinolones. In order to confirm the hypotheses put forth in this paper, the identification of antimicrobial resistance determinants and integrons that are associated with *armA* or *rmtB* on specific plasmid backbones should be conducted.

Notably, there was an increase in the proportion of amikacin-resistant isolates to total isolates in 2005 and 2006. It appears that most of the increase was due to an increase in the proportion of amikacin-resistant *K. pneumoniae* isolates to total *K. pneumoniae* isolates in 2005 and 2006 (around 18 to 20% of total isolates in 2005 and 2006 versus about 11% of total isolates in 2004 and about 6% of total isolates in 2003). Indeed, among the 139 *Enterobacteriaceae* isolates carrying *armA* or *rmtB* in 2005 and 2006, 98 (70.5%) isolates were *K. pneumoniae* and, surprisingly, 94 (95.9%) of the 98 *K. pneumoniae* isolates carried one of the following plasmids: *armA*-carrying Inc(unidentified), IncL/M, and IncFIIAs plasmids and an *rmtB*-carrying IncA/C plasmid, all of which were assumed to carry additional ESBLs and/or pACBLs. Therefore, an increase in the rate of resistance to amikacin in *K. pneumoniae* isolates in 2005 and 2006 seemed to result from the dissemination of *armA* and *rmtB*, which was mediated by the four kinds of conjugative plasmids that were assumed to carry additional ESBLs and/or pACBLs. Because ESBLs and pACBLs are prevalent among *K. pneumoniae* isolates in South Korea (11, 15), the association of *armA* and *rmtB* with ESBLs and/or pACBLs on conjugative plasmids might have contributed to the increase in the presence of *armA* and *rmtB* among *K. pneumoniae* strains in recent years.

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